

Serial No.: 09/972,756
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Entry of this amendment is respectfully requested. The amendment is made in compliance with 37 C.F.R. § 1.821-1.825. A paper copy of the above-named Sequence Listing, SEQUENCE IDENTIFICATION NUMBERS 1-25, accompanies this amendment. Also enclosed with the amendment is a letter requesting that the Examiner use the computer readable form of the Sequence Listing filed in a previous application.

This amendment contains no new matter. Applicant submits that this amendment, the accompanying paper copy of the Sequence Listing and letter requesting use of a previously filed computer readable form of the Sequence Listing serve to place this application in a condition of compliance with the rules 37 C.F.R. § 1.821-1.825.

Please direct any calls in connection with this application to the undersigned at (415) 781-1989.

Respectfully submitted,
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Filed under 37 C.F.R. § 1.34(a)

VERSION WITH MARKINGS TO SHOW CHANGES MADE

IN THE SPECIFICATION:

Paragraph beginning at page 8, line 31, has been amended as follows:

4. BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1. (A) Top panel, amino acid sequence comparison of the prototypic ISDR of NS5A from IFN-resistant HCV strain J (SEQ ID NO: 20) and the corresponding region of NS5A from HCV strains 1a (SEQ ID NO:21) and 1b which were used in this study. Bottom panel, structural representation of HCV NS5A representing the 447 aa protein from HCV-1a and -1b strains contained in our NS5A constructs. The ISDR is indicated in black, and is deleted from the ISDR construct derived from HCV-1a. Amino acid positions within NS5A are shown in bold type, those corresponding to positions within the HCV polyprotein are shown in normal type.

Paragraph beginning at page 57, line 15, has been amended as follows:

6.1 MATERIALS AND METHODS

Plasmid construction: To generate wild type HCV 1aNS5A constructs, the complete NS5A coding region from pSPns% (HCV-1a) was amplified by PCR using the oligonucleotide primers A, 5'-GGAATTCGAGCTCGCCCG (SEQ ID NO:1) and B, 5'-GCTCTAGAAGCACACGACATCTTC (SEQ ID NO:2) (*Eco*RI and *Xba*I sites underlined). The resulting product was directly cloned into pCR2.1 (Invitrogen) to yield plasmid pNS5A/CR2.1. The NS5A coding region was removed from pNS5A/CR2.1 as an *Eco*RI fragment for insertion in pBD (Stratagene) to yield pBD-NS5A, or as an

EcoRI-XbaI fragment for insertion into pcDNA3.1/His and pYES2 (Invitrogen), to give pcDNA3.1/His-NS5A and pYES2-NS5A, respectively. To generate the ISDR deletion mutant of NS5A, individual N-terminal and C-terminal coding fragments, each of which lacked the ISDR were generated. The N-terminal region, encoding amino acids 1-236, was amplified by PCR using primer A and primer C, 5'-CCACTCGAGCGGACAGTTGGCTGG (SEQ ID NO: 3) (*XhoI* site underlined). The C-terminal region, encoding amino acids 278-447, was amplified using primer B and primer D, 5'-CCGCTCGAGTGGTGATTCTGGTCTC (SEQ ID NO:4) (*XhoI* site underlined). The resulting PCR products were cloned directly into pCR2.1 to yield pN/CR2.1 and pC/CR2.1, respectively. The N-terminal coding region of NS5A was then removed from pN/CR2.1 for insertion into pcDNA-3, 1/His to generate pcDNA3.1/His-NS5A 1-236. An ISDR deletion construct was prepared by insertion of the C-terminal coding fragment into the *XhoI* and *XbaI* sites of pcDNA3.1/His-NS5A 1-236 fused in-frame with amino acids 278-447, deleting the entire ISDR. The insert from pcDNA3.1/His-ΔISDR was then subcloned into the 2μ yeast expression vectors pBD and pYES2 to give pBD-ΔISDR and pYES2-ΔSIDR, respectively. To obtain the NS5A coding region from HCV-1b, viral RNA was extracted from 100 μl of serum (Chomczynski and Sacchi, 1987, *Anal. Biochem.* 162:156-159) obtained under informed consent from a genotype 1b patient who failed to respond to IFN therapy. Response to IFN was determined by RT-PCR and bDNA assay, as previously described (Gretch et al., 1993, *J. Clin. Micro.* 31:289-291). Verification of HCV genotype was determined by a combination of RFLP and genotype-specific PCR analyses of the viral 5' untranslated region and sequences encoding the core protein, respectively (Davidson et al., 1995, *J. Gen. Virol.* 76:1197-1204; Okamoto et al., 1992, *J. Gen. Virol.* 73:673-679). Viral cDNA was synthesized by reverse transcription using the priming oligonucleotide 5' GTGGTGACGCAGCAGAGAGT (SEQ ID NO:5) (corresponding to nt 7681-7700 of HCV-J (Bukh et al., 1995, *Semin. Liver Dis.* 15:41-63), followed

with first-round PCR by addition of the upstream primer 5' CAGCCTCACCATCACTCAGC (SEQ ID NO:6) (corresponding to HCV-J nt 6256-6275). For directional cloning of NS5A, first-round PCR products were further amplified using the NS5A-specific nested-set oligonucleotide primer pair 5' CCTTCCATGGGCTCCGGCTCGTGGCTAAAG (SEQ ID NO:7) and 5' ATCGGATCCTTAGGACATTGAGCAGCAGACGA (SEQ ID NO:8) (*Nco*I and *Bam*HI sites underlined, respectively). After restriction enzyme digestion, the purified PCR products were cloned into the corresponding sites of pACT2 (Clontech) to give pAD-NS5A which encodes an AD-NS5A fusion protein corresponding to HCV-1b. While the relationship between the ISDR aa sequence of HCV-1a and IFN sensitivity has not been precisely determined, this region of NS5A (aa 237-276) possessed significant aa identity to the prototypic IFN-resistant ISDR sequence defined previously (Enomoto et al., 1996, *N. Engl. J. Med.* 334:77-81; Enomoto et al., 1995, *J. Clin. Invest.* 96:224-230) and present in our HCV-1b NS5A clone (Fig. 1A). Construction of PKR plasmids PBD-PKR K296R, pAD-PKR aa constructs K296R, 1-242, 244-551, 244-366, 376-551 and pAD-P58^{1PK} wt were described previously (Gale, Jr. et al., 1996, *Mol. Cell. Biol.* 16:4172-4181). pBD-PKR 99-551 was constructed by recovering the 1.6 kb *Nde*I/*Bam*I fragment from pET11A-PKR M7 (Barber et al., 1995 *Mol. Cell. Biol.* 15:3188-3146) and cloning it into the corresponding sites of pGBT10 (Gale, Jr. et al., 1996, *Mol. Cell. Biol.* 16:4172-4181) pEMBLYex4-K3L contains the entire vaccinia virus *K3L* gene inserted into pEMBLYex4 and is described in a separate manuscript. GST-NS5A was produced by introducing the *Bam*HI fragment from the HCV-1a NS5 clone pSPns5 into the plasmid pGEX2T (Smith and Johnson, 1988, *Gene* 67:31-40) to give pGST-NS5A. This construct encodes NS5A aa 1-427 fused in frame with the glutathione-S-transferase protein. The nt sequences of all constructs used in this study were confirmed by double stranded DNA sequence analysis using an Applied Biosystems automated sequencer.

Paragraph beginning at page 68, line 9, has been amended as follows:

Site-directed mutagenesis (Chameleon Double-Stranded Site-Directed Mutagenesis Kit; Stratagene) was used to introduce ISDR mutations corresponding to IFN-sensitive strains of HCV-1b into pBD-NS5A 1b-wt. Mutagenesis reactions were carried out as described by the manufacturer using the mutagenic primers shown in Table 3 (lower) (SEQ ID NOS:18-19). Template DNA was denatured by incubation at 100°C for 5 minutes, followed by annealing of the indicated mutagenic primer and the *Scal* to *Stul* selection primer 5' GTGACTGGTGGAGGCCTCAACCAAGTC (SEQ ID NO:9) (*Stul* restriction site underlined). T7 DNA polymerase-primer extension products were ligated and selected for the primer-encoded mutation(s) by digestion with *Scal* restriction enzyme and subsequent transformation into *XlmutS E. coli* (Stratagene). By this method, a set of isogenic NS5A constructs were constructed identical to NS5A 1b-wt except for the defined mutations introduced into the ISDR (see Table 1) (SEQ ID NOS: 20-25). pBD-NS5A 1b-2 and pBD-NS5A 1b-4 were generated directly from PBS-NS5A 1b-wt and encode a single (A2224V), or multiple (P2209L, T2214A, and T2217G) ISDR as mutations, respectively (Table 1). pBD-NS5A-5 encodes the ISDR as mutations P2209L, T2214A, T2217G and A2224V, and was produced by introducing an A2224V mutation into pBD-NS5A-4.

Paragraph beginning at page 86, line 1, has been amended as follows:

Table 1. ISDR sequence and corresponding IFN sensitivity of isogenic NS5A expression constructs.

Name ^a	ISDR Sequence (aa 2209-2248)	IFN Response ^b	Reference
1b-pt	PSLKATCTTHDSPDADLIEANLLWRQEMGGNITRVESEN (SEQ ID NO:20)	R	(Enomoto et al., 1996)
1b-wt	-----R----- (SEQ ID NO:22)	R	(Clements and Zink, 1996)
1b-2	-----R-----V----- (SEQ ID NO:23)	R/S	(Enomoto et al., 1996; Zeuzem et al., 1997)
1b-4	L----A--GR----- (SEQ ID NO:24)	S	
1b-5	L----A--GR-----V----- (SEQ ID NO:25)	S	(Enomoto et al., 1996)
1a-wt	-----AN-----E----- (SEQ ID NO: 21)	S	(Gale et al., 1997b)

^apt, HCV-J prototype reference sequence (GenBank™ Accession No. D90208); wt, wild type parental HCV-1b clone [GenBank™ Accession No. AF034151 (NS5A coding region only)].

^bR, IFN resistant; S, IFN sensitive; R/S, independently reported as IFN-sensitive in separate studies. The IFN-response phenotype corresponding to 1b-4 has not been determined. However, based upon published studies (Enomoto et al., 1996; Kurosaki et al., 1997) we predict this sequence to be associated with sensitivity to IFN.

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Paragraph beginning at page 88, line 1, has been amended as follows:

Table 3. Growth properties of cells expressing NS5A.

Construct ^a	Sense ^b	Antisense	nt
NS5A 1b-wt 1973-2419	5'TAAGCTTATGGGCTCCGGCTCGT GGCT (SEQ ID NO:10)	5'CAAGCTTGGATCCTTAGGACATTG AGC (SEQ ID NO:14)	6260-7598
NS5A 1973-2208	5'CATATGGGCTCCGGCTCGTGGCT A (SEQ ID NO:11)	5'GTCGACCGCAGACAACTGGCTAGC TGA (SEQ ID NO:15)	6260-6965
NS5A 2209-2274	5'GAATTCCCTTCCTTGAAGGCAAC ATGC (SEQ ID NO:12)	5'ATCGGATCCTTATACACCTTATTC TCTGA (SEQ ID NO:16)	6966-7165
NS5A 2180-2551	5'CCTTCCATGGCCCACATTACAGC AGAGACG (SEQ ID NO:13)	5'ATCGGATCCTTATACACCTTATTC TCTGA (SEQ ID NO:17)	6877-7094

Construct	Mutagenic Primer ^c	Mutation
NS5A 1b-2 NS5A 1b-5	5'GACTCCCCAGATGTTGACCTCATC (SEQ ID NO:18)	A2224V
NS5A 1b-4	5'TTGTCTGCGCTTTCCTTGAAGGCAGCATGCACTGGCCG TCACGAC (SEQ ID NO:19)	P2209L, T2214A, T2217G

^aaa and nt positions correspond to the PCR-amplified region. Numbering is based on the prototypic HCV-J sequence (Kato et al., 1990).

^bUnderlined sequence denotes cloning restriction site (described in text).

^cUnderlined codons correspond to the indicated aa mutations.

The enclosed text entitled "SEQUENCE LISTING" was inserted into the specification.